



Profiling *Vaccinium macrocarpon* components and metabolites in human urine and the urine *ex-vivo* effect on *Candida albicans* adhesion and biofilm-formation

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ABSTRACT

The aim of this work was to profile, by using an HPLC-MS/MS method, cranberry compounds and metabolites found in human urine after ingestion of a highly standardized cranberry extract (Anthocran®). Two different strategies were adopted for the data analysis: a targeted and an untargeted approach. These strategies allowed the identification of 42 analytes including cranberry components, known metabolites and metabolites hitherto unreported in the literature, including six valerolactones/valeric acid derivatives whose presence in urine after cranberry consumption has never been described before. Absolute concentrations of 26 over 42 metabolites were obtained by using pure available standards. Urine collected at different time points after the last dosage of Anthocran® were tested on the reference strain *C. albicans* SC5314, a biofilm-forming strain. Fractions collected after 12 h were found to significantly reduce the adhesion and biofilm formation compared to the control ($p < 0.05$). A similar effect was then obtained by using Anthocran™ Phytosome™, the lecithin formulation containing 1/3 of standardized cranberry extract and formulated to enhance the absorption of the cranberry components. The urinary profile of cranberry components and metabolites in the urine fractions collected at 1 h, 6 h and 12 h after the last capsule intake were then reproduced by using the pure standards at the concentration ranges found in the urine fraction, and tested on *C. albicans*. Only the mixture mimicking the urinary fraction collected at 12 h and containing as main components, quercetin and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone was found effective thus confirming the *ex-vivo* results.

1. Introduction

Candida albicans is one of the most common fungi causing disease in humans and the most frequently isolated fungal pathogen in nosocomial urinary tract infections (UTIs) [1,2]. Urological devices, urological procedures, diabetes and being female are the main factors linked to candiduria [3]. Catheters, which are used in up to 20% of hospitalized subjects [4], represent an adhesion substrate for microorganisms that can easily develop biofilm on plastic or silicone surfaces. The most important feature of microbial biofilms is their tolerance to

antimicrobial therapies [5], leading to recurrent or persistent infections. Therefore, alternative approaches to conventional antifungal therapy are desirable and among these the search of botanical products provides opportunities for new therapeutic approaches.

Cranberry (*Vaccinium macrocarpon*) is a rich source of polyphenols, which possess beneficial properties towards pathogenic infections including urinary tract infections (UTIs), dental caries and stomach ulcers [6]. Moreover, berry phenolics showed antioxidant, anti-inflammatory and anticancer properties [7,8]. A synergy of all the phytochemicals could explain the great health benefits of cranberry reported *in vitro*

Abbreviations: UTIs, urinary tract infections; PACs, proanthocyanidins; CFM-ID, competitive fragmentation modeling for metabolite identification

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studies [9–11]. Despite all these potential applications, thus far prevention of UTIs remains the main application for cranberry based products [12]. The major constituents of cranberry are flavonols, anthocyanins, proanthocyanidins (PACs), flavan-3-ols and phenolic acids and derivatives [13]. Several *in vitro* studies have supported the hypothesis that the antiadhesive properties of cranberry are due to PACs, and in particular to the A-type [14]. However, controversial results regarding their presence in human urine are reported: many *in vivo* studies have demonstrated that they are not detected after cranberry intake [15–19], but two works have shown their presence in urine at a very low concentration [20,21]. The use of different dosages and non-standardized cranberry products could explain these controversial results. The purpose of the present work is to profile the components and metabolites in human urine after ingestion of a highly standardized cranberry extract (Anthocran®, Indena S.p.A.) which has been found effective in human studies [22–24]. Furthermore, the evaluation of the activity of urinary fractions on *C. albicans* adhesion collected at different times following cranberry intake was performed. The quantitative analysis of the metabolites identified in each urine fraction combined with the urine activity on *C. albicans* adhesion has permitted the identification of an array of compounds responsible for inhibiting fungal adherence. Finally, the *ex-vivo* activity of Anthocran™ Phytosome™, lecithin formulation of the standardized cranberry extract was tested in order to evaluate whether the lipid matrix can improve the bioavailability and bioactivity of the extract.

2. Materials and methods

2.1. Reagents

Formic acid, ethyl gallate, protocatechuic acid, p-coumaric acid, gallic acid, sinapinic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 3-(4-hydroxyphenyl)-propionic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, 3,4-dihydroxyhydrocinnamic acid, 2-hydroxyhippuric acid, quinic acid, 2-methylhippuric acid, YPD medium, Roswell Park Memorial Institute 1640 medium (RPMI), phosphate buffered saline (PBS), crystal violet, methanol and LC-MS grade solvents were purchased from Merck KGaA, Darmstadt, Germany. Kaempferol, quercetin, syringetin, quercetin-3-O-rhamnoside, quercetin-3-O-galactoside were from Extrasynthese (Genay Cedex, France). Quercetin-3-O-arabinofuranoside, 3-hydroxyhippuric acid and 4-hydroxyhippuric acid were from Carbosynth (Compton Berkshire, UK). LC-grade H₂O (18 MΩ cm) was prepared with a Milli-Q H₂O purification system (Millipore, Bedford, MA, USA). SPE Hypersep C18 column (100 mg/mL) were from Thermo Scientific (Milan, Italy). Standardized cranberry extract (*V. macrocarpon*) and the capsules containing 36 mg PACs/capsule (Anthocran®), 12 mg PACs/capsule Anthocran™ Phytosome™ and placebo capsule were supplied by Indena S.p.A (Milan, Italy).

2.2. Synthesis of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (I)

¹H NMR spectra were recorded operating at 300 MHz while ¹³C NMR at 75.43 MHz. Chemical shifts are reported in ppm relative to residual solvent (CHCl₃ or DMSO) as internal standard. Signal multiplicity is designed according to the following abbreviations: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet, br s = broad singlet, br t = broad triplet. Purifications were performed by flash chromatography using silica gel (particle size 40–63 μm, Merck) on Isolera™ (Biotage, Uppsala, Sweden) apparatus.

Palladium on carbon, 3,4-bis(benzyloxy)benzaldehyde, 2(5H)-furanone, *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMSOTf), 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), sodium bisulfite, 37% HCl, cyclohexane, ethyl acetate, tetrahydrofuran, ethanol and methanol were purchased from Merck KGaA, Darmstadt, Germany.

Compound I was afforded (Fig. 1) by Mukaiyama aldol addition

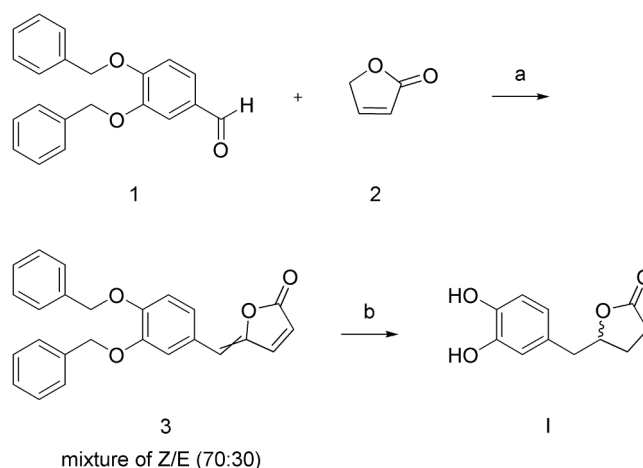


Fig. 1. Synthesis of compound I – a) DBU, TBDMSOTf, THF dry, –10 °C; b) H₂, Pd/C, CH₃OH, RT.

between 3,4-bis(benzyloxy)benzaldehyde (1) and 2(5H)-furanone (2) in presence of *tert*-butyldimethylsilyl trifluoromethanesulfonate and DBU. 5-(3',4'-bis(benzyloxy)benzylidene)furan-2(5H)-one (3) was obtained as a mixture of Z/E isomers (70:30). Reduction of 3 by H₂ Pd/C provided the final compound 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (I) as racemic mixture.

5-(3',4'-Bis(benzyloxy)benzylidene)furan-2(5H)-one (3). Under nitrogen atmosphere, DBU (0.28 mL, 1.88 mmol) was added dropwise to a solution of 2 (158 mg, 1.88 mmol) in dry THF (16 mL). The mixture was stirred for 30 min at room temperature. After cooled down to –10 °C *tert*-butyldimethylsilyl trifluoromethanesulfonate (0.48 mL, 2.07 mmol) and 1 (600 mg, 1.88 mmol) were added dropwise and the mixture was stirred 1 h at –10 °C, then DBU (0.56 mL, 3.76 mmol) was added dropwise. The reaction mixture was stirred overnight at room temperature, then solvent was removed under vacuum. Ethyl acetate (20 mL), EtOH (5 mL) and saturated solution of sodium bisulfite (5 mL) were added to the crude residue and stirred overnight at 40 °C. The phases were separated and the organic layer was diluted with ethyl acetate (15 mL), treated with 2.9 N HCl (3 × 20 mL), washed with brine (20 mL), dried and concentrated to afford a sticky black oil. The crude product was purified on silica gel (75:25 cyclohexane/ethyl acetate) to afford the title compound as an orange/brown oil (137 mg, 0.36 mmol, 19% yield).

¹H NMR (300 MHz, CDCl₃) Z isomer: δ = 7.53 (d, *J* = 7.0 Hz, 4H), 7.49–7.24 (m, 9H), 6.92 (d, *J* = 8.4 Hz, 1H), 6.15 (d, *J* = 5.3 Hz, 1H), 5.91 (s, 1H), 5.22 (s, 2H), 5.20 (s, 2H).

¹H NMR (300 MHz, CDCl₃) E isomer: δ = 7.53 (d, *J* = 7.0 Hz, 4H), 7.49–7.24 (m, 9H), 6.92 (d, *J* = 8.4 Hz, 1H), 6.21 (d, *J* = 5.3 Hz, 1H), 5.91 (s, 1H), 5.18 (s, 2H), 5.14 (s, 2H).

5-(3',4'-dihydroxyphenyl)-γ-valerolactone (I). 3 (275 mg, 0.72 mmol) was dissolved in CH₃OH (16.50 mL), and 3% Pd/C (40 mg) was added. The reaction mixture was stirred overnight under hydrogen atmosphere at room temperature, then the catalyst was removed by filtration. The filtrate was concentrated in vacuo to afford the desired product as an orange oil (125 mg, 0.60 mmol, 83% yield).

¹H NMR (300 MHz, CD₃OD) δ = 6.71–6.67 (m, 2H), 6.56 (dd, *J* = 7.9, 2.2 Hz, 1H), 4.77–4.66 (m, 1H), 2.87 (dd, *J* = 14.1, 6.1 Hz, 1H), 2.78 (dd, *J* = 14.0, 6.1 Hz, 1H), 2.52–2.41 (m, 1H), 2.35 (dd, *J* = 9.4, 4.7 Hz, 1H), 2.29–2.17 (m, 1H), 2.03–1.88 (m, 1H).

¹³C NMR (75 MHz, CD₃OD) δ = 173.17, 144.88, 143.80, 127.60, 120.45, 116.20, 114.90, 81.86, 40.03, 28.14, 26.44.

2.3. Subject selection

A total of thirteen volunteers (mean age 25 ± 4 years and BMI 20.6 ± 2.0 kg m^{–2}) were recruited from students and staff of the

University of Milan according to the following inclusion criteria: women, 18–40 years of age, normal weight for height ($18\text{--}25\text{ kg m}^{-2}$), non-smokers, no history of cardiovascular, diabetes, hepatic, renal, or gastrointestinal diseases. Subjects with allergy and/or aversion to cranberry and/or cranberry products were excluded. Other exclusion criteria were as follow: consumption of any dietary supplement, drug or medication for at least one month before the beginning of the study. The study was performed in accordance with the ethical standards established in the 2013 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan (December 18, 2018, ref. 57/18). The study was registered at www.isrctn.org as ISRCTN32556347. All participants signed an informed consent form.

2.4. Study design on healthy volunteers

Subjects were instructed to limit the consumption of polyphenols at least 72 h before experimentation and during the trial. A list of foods to be avoided has been provided to the volunteers. The list included: fruits and vegetables rich in polyphenols (e.g. berries, red/purple fruits/vegetables), chocolate and some beverages such as coffee, tea, wine and fruit juice. The study consisted of a randomized, double blind, 2-arm repeated measure cross-over design. One group of subjects consumed 2 capsules of Anthocran® per day, while the other group 2 the capsules of Anthocran™ Phytosome™ per day. The experiment was 7-day long. Urine samples were collected before starting supplementation (day 1, time 0) and after the last dosage (day 7) at the following time-points: 1, 2, 4, 6, 10, 12, 24 h. After one week of wash-out the groups inverted the treatment. Each subject received a box containing the number of capsules to consume during the experiment. Capsules were provided in a blind condition. Subjects were instructed to swallow two capsules per day, the first one in the morning before breakfast and the second one before dinner with a glass of water. Three volunteers consumed two placebo capsules with the same shape, size, colour, flavour and excipients of the products tested. Urine samples were collected at the same time-points as previously reported and used as control to verify the influence of diet and circadian rhythm on *C. albicans* activity. Ethyl gallate 10 μM was added as internal standard in the sample used for the MS analysis and all the samples were stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

2.5. Sample preparation

An aliquot (1 mL) of each of the 10 subjects' urine was centrifuged at $10000 \times g$ for 5 min and the supernatant was extracted on the SPE column, working at 1 mL/min. Salts were removed with water and then all the compounds retained were eluted with 1 mL 100% acetonitrile. The fractions collected were dried under vacuum and then solubilized in 100 μL $\text{H}_2\text{O}-\text{CH}_3\text{OH}-\text{HCOOH}$ (90:10:0.1, v/v). For the quantitative analysis, the stock solutions of the standards were prepared in methanol and then diluted in a pool of the pre-treatment urine samples to obtain the final concentrations for each calibration curve. The samples were then added with ethyl gallate 10 μM as internal standard and processed as described.

2.6. Chromatographic conditions

Cranberry components and urine metabolite separation was performed on a reversed-phase Agilent Zorbax SB-C18 column ($150 \times 2.1\text{ mm}$, i.d. $3.5\text{ }\mu\text{m}$, CPS analitica, Milan, Italy), protected by an Agilent Zorbax guard column, kept at $40\text{ }^{\circ}\text{C}$, by an UltiMate 3000 system (Dionex) equipped with an autosampler kept at $4\text{ }^{\circ}\text{C}$ working at a constant flow rate (200 $\mu\text{L}/\text{min}$). Each sample (10 μL) was injected into the column and both cranberry components and urine metabolites were eluted with an 80 min multistep gradient of phase A $\text{H}_2\text{O}-\text{HCOOH}$ (100:0.1, v/v) and phase B $\text{CH}_3\text{CN}-\text{HCOOH}$ (100:0.1, v/v): 0–45 min, from 10% B to 20% B; 45–65 min, from 20% B to 60% B; 65–66 min, from 60% B to 90% B; 66–70 min, isocratic of 90% B; 70–71 min, from

90% B to 10% B, and then 71–80 min of isocratic 10% B.

2.7. Polyphenol class identification by HPLC-UV analysis

The identification of polyphenol classes was carried out by HPLC-UV analysis on a HPLC Surveyor LC system (Thermo Fisher Scientific, Milan, Italy) equipped with a quaternary pump, UV-VIS detector (PDA) and an autosampler. The scan range was set from 200 nm to 600 nm. A solution of 4 mg/mL of cranberry extract in $\text{H}_2\text{O}/\text{CH}_3\text{OH}/\text{HCOOH}$ (90/10/0.1% v/v) was used for the analysis.

2.8. Cranberry component profiling and urine metabolite characterization by high resolution mass spectrometry

Each sample (10 μL) was injected into the RP column as previously described: the cranberry extract was analyzed at a concentration of 4 mg/mL in $\text{H}_2\text{O}-\text{CH}_3\text{OH}-\text{HCOOH}$ (90:10:0.1, v/v), while the urine samples were analyzed after the treatment described in section 2.3. The analyses were performed on a LTQ-Orbitrap XL mass spectrometer using an ESI source. Mass spectra were acquired in positive and in negative ion modes. A list of 20 background ions was adopted as lock mass values for real time mass calibration [25]. The source parameters used for the positive mode are: spray voltage 4 kV, capillary temperature $300\text{ }^{\circ}\text{C}$, capillary voltage 30 V, tube lens offset 90 V; for the negative ion mode: spray voltage 4 kV, capillary temperature $300\text{ }^{\circ}\text{C}$, capillary voltage -23 V , tube lens offset -140 V . The instrument was set up to work in a data-dependent scan mode to acquire both full MS and MS/MS spectra. Full MS spectra were acquired in profile mode by the FT analyzer in a scan range of m/z 100–1200, using AGC scan target 5×10^5 and resolution 30,000 FWHM at m/z 400. Tandem mass spectra were acquired by the linear ion trap (LTQ) which was set up to fragment the 3 most intense ions exceeding 1×10^4 counts. Mass acquisition settings were: centroid mode, AGC scan target 5×10^4 , precursor ion isolation width of m/z 3, and collision energy (CID) of 35 eV. Dynamic exclusion was enabled to reduce redundant spectra acquisition: 2 repeat counts, 20 sec repeat duration, 30 sec of exclusion duration. Moreover, only singly and unassigned charged ions were fragmented. Instrument control and spectra analysis were provided by the software Xcalibur 2.0.7 and Chromeleon Xpress 6.80.

2.9. Targeted and untargeted analyses of cranberry components and metabolites in human urine

An in-house database was created for the targeted analysis by adding all the characterized cranberry extract components as well as known cranberry metabolites identified in other studies and cranberry components deriving from other cranberry sources even if not present in the extract under investigation. The identification was carried out on the QualBrowser tool of Xcalibur 2.0.7 by using the accurate mass and the isotopic and fragmentation patterns.

The untargeted analysis consisted of searching for all the ions present in the urine samples collected after the cranberry consumption that were not present or present at intensity relative to noise ($< 5 \times 10^2$ counts) in the pre-treatment sample. Spectra analyses were carried out on the QualBrowser tool of Xcalibur 2.0.7 by screening the full MS spectra acquired in negative ion mode in mass ranges of m/z 5 with 10 min as acquisition time for each sample. Each ion detected with these filters was exported with the relative MS/MS spectrum, if present. Identification was performed by following two different approaches based on the accurate mass and isotopic and fragmentation patterns. The first approach consists of giving the precursor ion and the MS/MS spectrum list as inputs in the Compound Identification tool of CFM-ID [26], using as mass tolerance error 10 ppm for the precursor ion and 0.3 Da for the fragments. CFM-ID performs a search for candidates in available databases (HMDB and KEGG) based on the accurate mass, then generating *in-silico* MS/MS spectra of all the candidates and then

comparing the experimental data with those obtained *in-silico*. The top candidates were ranked (Jaccard Score) according to how closely they matched and returned to a list. The second approach was initially focused on the calculation of the elemental composition performed on the Elemental Composition page of Xcalibur 2.0.7 by using the following parameters: mass tolerance 10 ppm, charge -1 , C, H, O, N, P, S as elements in use. The top 5 formulae were searched in available databases such as PubChem, METLIN, MassBank and in the literature in order to obtain a list of candidates. Following this, the Peak Assignment tool of CFM-ID was used to predict the MS/MS spectra of the putative identified compounds and to compare the *in-silico* spectra obtained with the experimental spectra.

2.10. Quantitative analysis

The calibration curves for each available metabolite were built by plotting the peak area ratios of metabolite/ethyl gallate versus the nominal concentrations of the metabolite by weighted ($1/x^2$) least-squares linear regression. Table 1 shows all the obtained linear curves and the relative limit of quantification (LOQ). All the samples and calibration solutions were analyzed in triplicates. The areas under the curve of the extracted ion chromatogram of each identified metabolite was integrated by using the Genesis peak algorithm of the Qual Browser tool of Xcalibur 2.0.7.

2.11. *Candida albicans* biofilm formation assay

The biofilm-forming ability of *C. albicans* under various conditions (i.e. medium supplementation with cranberry extract or urine fractions) was evaluated on polystyrene 96-well plates using the reference strain *C. albicans* SC5314 [27]. Prior to experiments, *C. albicans* was grown overnight in yeast extract, peptone, dextrose (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) broth (YPD) at 30 °C in an orbital shaker. Cells were then harvested and washed with cold phosphate-buffered saline (PBS) and counted by hemocytometer. A standard inoculum of 5×10^5 yeast cells/mL was prepared in Roswell Park Memorial Institute 1640 medium (RPMI) and incubated in presence and absence of urine fractions as well as in the presence of Anthocran™

0.1 mg/mL. 100 μ L/well of the inoculum was firstly incubated at 37 °C for 1 h to promote adhesion. Non-adherent cells were removed and wells washed with PBS; medium was replaced and the plate further incubated for 24 h. Biofilm-forming ability was quantified by crystal violet (CV) staining for total biomass measurement [28]. Two independent experiments were carried out with six replicates for each condition. The statistical analysis was performed by GraphPad Prism 6.02 for Windows, GraphPad Software, La Jolla California USA, (www.graphpad.com) using the one-way ANOVA with Dunnett's multiple comparisons test.

3. Results and discussion

3.1. Compliance

Volunteers confirmed the consumption of the capsules and the compliance was also verified by counting the capsules in the returned boxes. All the participants had 100% compliance and declared no adverse effects following the intervention.

3.2. Characterization of Anthocran® components

Polyphenol classes of the cranberry extract were characterized by HPLC-UV analysis at typical wavelength: 310–320 nm for phenolic acids, 350–370 nm for flavonols, 520 nm for anthocyanins, 278 nm for benzoic acids, flavanols and PACs. The identification of each compound in the extract was then obtained on the basis of the accurate mass and of the isotopic and the fragmentation patterns, by acquiring the mass spectra in positive and in negative ion mode. All identified compounds are reported in Table 2, Tables 3 and 4.

3.3. Targeted analysis of human urine after cranberry extract intake

The targeted analysis consisted of searching in the urine samples for the compounds listed in an in-house database (total number of compounds = 138), which comprises the cranberry extract components characterized as reported in section 3.2 and cranberry compounds and metabolites as reported elsewhere [15–21,29,30]. Identification was

Table 1
Calibration curve parameters for the available metabolites.

Metabolite	[M – H] [–]	Slope	Intercept	R ²	Limit of Quantification (μ M)
Protocatechuic acid	153.0189	0.1529	–0.04789	0.991	0.25
p-Coumaric acid	163.0408	0.2786	–0.03032	0.995	0.01
Gallic acid	169.0146	0.0253	–0.00585	0.991	1
Sinapinic acid	223.0608	0.0452	–0.00523	0.990	0.005
Kaempferol	285.0407	1.0160	0.07999	0.991	0.001
Quercetin	301.0353	1.1520	–0.05015	0.999	0.005
Syringetin	345.0618	0.6626	0.00924	0.981	0.005
Quercetin-3-O-arabinofuranoside	433.0731	0.7625	–0.01439	0.997	0.0025
Quercetin-3-O-rhamnoside	447.0920	0.9010	–0.02547	0.997	0.001
Quercetin-3-O-galactoside	463.0884	0.5151	–0.02053	0.996	0.0025
2-hydroxybenzoic acid	137.0243	1.1470	–0.13480	0.998	0.25
3-hydroxybenzoic acid	137.0231	0.1113	–0.01402	0.996	0.05
4-hydroxybenzoic acid	137.0232	0.1204	–0.04054	0.999	1
2,3-dihydroxybenzoic acid	153.0201	1.2380	–1.89800	0.984	1.5
2,5-dihydroxybenzoic acid	153.0194	0.2438	0.67780	0.998	5
2,4-dihydroxybenzoic acid	153.0175	0.3176	–0.33540	0.989	1.5
3-(4-hydroxyphenyl)-propionic acid	165.0561	0.0048	0.00106	0.999	0.25
3,4-dihydroxyphenylacetic acid	167.0349	0.0228	0.05486	0.981	1.5
Hippuric acid	178.0509	0.3032	120.80000	0.993	1
3,4-dihydroxyhydrocinnamic acid	181.0506	0.2474	0.37710	0.997	0.25
p-Hydroxyhippuric acid	194.0450	0.2756	10.42000	0.995	1
m-Hydroxyhippuric acid	194.0446	0.3605	–2.94400	0.991	1
o-Hydroxyhippuric acid	194.0461	0.6801	–4.23000	0.996	1
2-methylhippuric acid	192.0670	0.7954	3.60500	0.999	1
Quinic acid	191.0554	0.01012	0.0097	0.999	0.05
5-(3',4'-dihydroxyphenyl)- γ -valerolactone	207.0666	0.2411	–0.47450	0.999	0.1

Table 2
Anthocyanins composition in Anthocran®.

Name	RT (min)	[M] ⁺	Fragments
Anthocyanins			
Cyanidin	19	287.0556	259 + 255 + 251 + 245 + 242 + 125
Peonidin	24	301.0712	268 + 258 + 230 + 177 + 151
Cyanidin-3-O-arabinoside	6.2	419.0978	287
Peonidin-3-O-arabinoside	10	433.1135	301
Cyanidin-3-O-galactoside	4.2	449.1084	287
Cyanidin-3-O-glucoside	4.6	449.1084	287
Petunidin-3-O-arabinoside	5	449.1084	317
Peonidin-3-O-galactoside	7.3	463.1240	301
Peonidin-3-O-glucoside	7.6	463.1240	301
Malvidin-3-O-galactoside	8	493.1346	331
Malvidin-3-O-glucoside	8.4	493.1346	331

Table 3
Phenolic acids in Anthocran®.

Name	RT (min)	[M – H] [–]	Fragments
Phenolic acids			
Benzoic acid	8.3	121.0290	77
Protocatechuic acid	4	153.0188	109
p-Coumaric acid	13.6	163.0395	119
Gallic acid	2.5	169.0137	125
Caffeic acid	7.9	179.0344	135
Ferulic acid	4.9	193.0501	149 + 134
Sinapinic acid	6.2	223.0607	179 + 164 + 149 + 135
Caffeoyl glucose	5	341.0873	179 + 135
Chlorogenic acid	6	353.0873	191 + 179 + 161

performed by considering the following parameters: accurate mass, isotopic pattern, MS/MS fragments and the retention time. In more details for each of the compound listed in the database, the SIC was reconstituted setting as filter ion the m/z calculated for the target compound; when a peak with the same RT of the targeted compound was found, the m/z of the parent ion, the isotopic pattern and MS/MS data were retrieved and compared with that of the standard. For some cranberry components (gallic acid, kaempferol, quercetin, syringetin, quercetin-3-O-arabinofuranoside, quercetin 3-O-rhamnoside and quercetin-3-O-galactoside) the intensity of the parent compound was not intense enough to perform CID experiments and their identity was confirmed by comparing the RT and isotopic pattern with those of genuine standards.

Tables 5 and 6 report the compounds and metabolites identified by using such an approach and setting the ion source in negative ion mode. Specifically, the identification of metabolites reported in Table 5 was confirmed by pure standards, while metabolites listed in Table 6 were putatively identified on the base of the accurate mass, isotopic pattern and, when present, fragmentation pattern. Analyses were also carried out in positive ion mode but no additional components were detected, nor were anthocyanins, which are characterized by an high response in such a polarity mode. The lack of detection of anthocyanins can be explained by considering their biotransformation mediated by the colonic microflora into small phenolic compounds, such as protocatechuic acid, phloroglucinaldehyde, ferulic acid, syringic acid, gallic acid, caffeic acid and vanillic acid [31–33], some of which were detected in the urine.

Some flavonols were found as well as some of their glycosylated forms such as kaempferol, quercetin and traces of its glycosides (quercetin-3-O-arabinofuranoside, quercetin-3-O-rhamnoside and quercetin-3-O-galactoside), syringetin and traces of isorhamnetin-3-O-arabinopyranoside. The low abundance of the glycosylated forms can be explained by considering the enzymatic hydrolysis into the corresponding aglycones mediated by cellular and bacterial β -glucosidases and occurring in the intestine [34]. The aglycones can be transformed by microbiota in the intestinal tract into phenolic acids by C-ring

cleavage and/or can undergo phase-II metabolism (i.e. glucuronidation) or methylation [35]. Metabolic studies performed on quercetin (the most representative flavonol) and related glycoside in the intestine showed that the main metabolites are represented by hydroxyphenylacetic acid catabolites [36,37]. In fact, 3,4-dihydroxyphenylacetic acid was found in urine as possible metabolite of flavonols deriving from intestinal microbiota metabolism.

Proanthocyanidins present in the extract (procyanidins A-type and B-type) were not detected in urine in the present study. Results from previous studies on this class of polyphenols are quite controversial, in particular concerning procyanidin A2, to which several studies attributed the activity of cranberry products in UTI prevention. In most cases [15–19], as in the present study, PACs were not detected in human urine while two works reported PACs in human urine: one work was performed on men and postmenopausal women of 50–70 years who took a single dose of 237 mL of cranberry juice (PACs content in the juice was not reported) [20]; in the second study, performed by the same research group, five young women (20–30 years) consumed 237 mL/day of cranberry juice (140 mg of PACs) according to a weekly schedule for 7 weeks [21]. The results that they obtained showed very low levels of PAC-A2 quantified in human urine ($C_{MAX} = 24$ ng/mg creatinine) and they concluded that PAC-A2 cannot be used as biomarker of cranberry intake since there was no correlation with the amount of juice consumed. Taking into consideration all these results, it is commonly accepted that PACs have a very low bioavailability, which decreases as the degree of their polymerization increases [38]. Moreover, it is reported that human microbiota degrades PACs in the colon into phenolic compounds: phenylacetic acids and phenylpropionic acids as metabolites of procyanidins A2, B2, catechin and epicatechin; for procyanidin B2, catechin and epicatechin, valerolactones and valeric acids derivatives have also been reported [39,40]. Although in the present paper the valerolactones origin has not been investigated, we can suggest they come from a microbiota-based transformation of cranberry catechin/epicatechin/PACs. This assumption is supported by independent researchers showing the catechin/epicatechin/PACs biotransformation to valerolactones is driven by gut microbiota. In particular, a metabolome study based on [2-¹⁴C] (–)-epicatechin in humans showed valerolactones as epicatechin metabolites [41]. Moreover, the bacteria *Eggerthella lenta* and *Flavonifractor plautii* were identified as responsible for catechin/epicatechin degradation to valerolactone derivatives by M. Kutschera et al. [42]. Li et al. described phenyl-valerolactones as the main tea catechin metabolites produced by gut microorganisms and detected in human urine and blood [43]. A gamma valerolactone was identified by Appeldoorn M.M. et al. as a main metabolite of procyanidin dimer metabolized by human microbiota [44]. Valerolactones were also detected in human urine by Ottaviani et al. after the consumption of flavanols and PACs [45].

In the present study, the following metabolites deriving from phenylpropionic acids [46] were detected: 3,4-dihydroxyphenylacetic acid, 3-(4-hydroxyphenyl)-propionic acid, dihydroxybenzoic acids,

Table 4
Flavanols, Flavanols and PACs in Anthocran®.

Name	RT (min)	[M + H] ⁺	Fragments	[M – H] [–]	Fragments
Flavanols, Flavanols, PACs					
Coumarin	14	147.0446	119 + 91	145.0289	–
Scopoletin	17.8	193.0501	165 + 152 + 133 + 119 + 105	191.0344	–
Kaempferol	55	287.0556	259 + 251 + 241 + 231 + 213 + 165 + 153 + 137 + 121	285.0399	–
Epicatechin	5.7	291.0869	169 + 165 + 151 + 147 + 139 + 123	289.0712	245 + 205 + 179 + 161 + 151 + 137 + 125 + 109
Catechin	9	291.0869	169 + 165 + 151 + 147 + 139 + 123	289.0712	245 + 205 + 179 + 161 + 151 + 137 + 125 + 109
Quercetin	48.5	303.0505	257 + 247 + 229 + 165 + 153 + 149 + 137 + 121	301.0348	273 + 257 + 229 + 179 + 151 + 121 + 107
Epigallocatechin	4.4	307.0818	–	305.0661	261 + 221 + 219 + 179 + 137 + 125
Gallocatechin	3.1	307.0818	–	305.0661	261 + 221 + 219 + 179 + 137 + 125
Isorhamnetin	55.8	317.0661	299 + 285 + 281 + 274 + 257 + 165 + 153 + 139	315.0505	287 + 271 + 259 + 243 + 203 + 163 + 151
Myricetin	32	319.0454	290 + 273 + 255 + 245 + 165 + 153 + 137	317.0298	255 + 227 + 193 + 179 + 151 + 137 + 107
3-O-methylmyricetin	50	333.0610	301 + 287 + 277 + 273 + 245 + 193 + 165 + 153 + 139	331.0454	287 + 271 + 263 + 179 + 151
Syringetin	55.8	347.0767	315 + 291 + 287 + 269 + 181 + 165 + 153 + 139	345.0610	315
Quercetin-3-O-arabinofuranoside	25.2	435.0927	303	433.0771	301
Quercetin-3-O-arabinopyranoside	28.4	435.0927	303	433.0771	301
Quercetin-3-O-xylopyranoside	26.6	435.0927	303	433.0771	301
Catechin-3-O-gallate	16.6	443.0978	291 + 273 + 151 + 139	441.0822	315 + 297 + 289 + 161 + 153
Epicatechin-3-O-gallate	20	443.0978	291 + 273 + 151 + 139	441.0822	330 + 305 + 289 + 161 + 139
Kaempferol-7-O-glucoside	27.2	449.1084	287	447.0927	284
Isorhamnetin-3-O-arabinofuranoside	37	449.1084	317	447.0927	314
Isorhamnetin-3-O-xylopyranoside	38.5	449.1084	317	447.0927	314
Isorhamnetin-3-O-arabinopyranoside	41	449.1084	317	447.0927	314
Quercetin-3-O-rhamnoside	30.5	449.1084	303	447.0928	301
Myricetin-3-O-arabinofuranoside	16.6	451.0877	319	449.0720	317
Myricetin-3-O-arabinopyranoside	19.6	451.0877	319	449.0720	317
Myricetin-3-O-xylopyranoside	19	451.0877	319	449.0720	317
Quercetin-3-O-glucoside	22.4	465.1033	303	463.0877	301
Quercetin-3-O-galactoside	21.5	465.1033	303	463.0877	301
Isorhamnetin-3-O-glucopyranoside	34.5	479.1189	317	477.1033	315
Isorhamnetin-3-O-glucopyranoside	32.8	479.1189	317	477.1033	315
Isorhamnetin-3-O-galactoside	31.1	479.1189	317	477.1033	315
Syringetin-3-O-arabinofuranoside	38.8	479.1189	347	477.1033	345
Syringetin-3-O-xylopyranoside	39.8	479.1189	347	477.1033	345
Syringetin-3-O-arabinopyranoside	43.5	479.1189	347	477.1033	345
Myricetin-3-O-glucoside	15.1	481.0982	319	479.0826	317
Myricetin-3-O-galactoside	14.5	481.0982	319	479.0826	317
Syringetin-3-O-rhamnoside	43.8	493.1346	347	491.1189	–
Proanthocyanidin A-type dimer	17/23	577.1346	437 + 425 + 397 + 287	575.1189	425 + 289 + 287
Proanthocyanidin B-type dimer	22.6	579.1502	453 + 439 + 427 + 409 + 301 + 291	577.1346	425 + 407 + 289
Proanthocyanidin A-type trimer	16/25/27.3	865.1980	713 + 577 + 425 + 287	863.1823	575 + 423 + 289
Proanthocyanidin B-type trimer	11.5/14.3/24	867.2136	579 + 427 + 409 + 291	865.1979	577 + 425 + 407 + 287

Table 5

Cranberry components and metabolites identified using the targeted analysis.

Metabolite identification	RT (min)	Calculated [M – H] [–]	Observed [M – H] [–]	MS/MS fragments	Delta ppm
Protocatechuic acid	4.1	153.0188	153.0189	109	–0.588
<i>p</i> -Coumaric acid	14.1	163.0395	163.0408	119	–8.035
Gallic acid	2.5	169.0137	169.0146	–	–5.325
Sinapinic acid	19.4	223.0607	223.0608	179 + 164 + 149	–0.717
Kaempferol	55	285.0399	285.0407	–	–2.877
Quercetin	48.9	301.0348	301.0353	–	–1.395
Syringetin	55.8	345.0610	345.0618	–	–2.289
Quercetin-3-O-arabinofuranoside	28.5	433.0771	433.0731	–	9.306
Quercetin 3-O-rhamnoside	30.6	447.0928	447.0920	–	1.789
Quercetin-3-O-galactoside	21.6	463.0877	463.0884	–	–1.684
2-hydroxybenzoic acid	24.2	137.0239	137.0243	93	–3.065
3-hydroxybenzoic acid	8.9	137.0239	137.0231	–	5.984
4-hydroxybenzoic acid	6.6	137.0239	137.0232	93	4.671
2,3-dihydroxybenzoic acid	9.1	153.0187	153.0201	109	–8.561
2,5-dihydroxybenzoic acid	6.8	153.0187	153.0194	109	–3.856
2,4-dihydroxybenzoic acid	8.6	153.0187	153.0175	109	8.169
3-(4-hydroxyphenyl)-propionic acid	12.1	165.0552	165.0561	–	–5.816
3,4-dihydroxyphenylacetic acid	4.5	167.0344	167.0349	–	–2.574
Hippuric acid	8.2	178.0504	178.0509	134	–2.640
3,4-dihydroxyhydrocinnamic acid	7.1	181.0499	181.0506	137 + 121	–3.811
<i>p</i> -Hydroxyhippuric acid	3.6	194.0456	194.0450	150 + 100 + 93	3.401
<i>m</i> -Hydroxyhippuric acid	4.5	194.0456	194.0446	150 + 100 + 93	5.256
<i>o</i> -Hydroxyhippuric acid (salicyluric acid)	14.1	194.0456	194.0461	150 + 100 + 93	–2.628
5-(3',4'-dihydroxyphenyl)- γ -valerolactone	12	207.0657	207.0659	163 + 122 + 109	–0.821

Table 6

Metabolites putatively identified using the targeted analysis.

Metabolite putative identification	RT (min)	Calculated [M – H] [–]	Observed [M – H] [–]	MS/MS fragments	Delta ppm
Isorhamnetin-3-O-arabinopyranoside	41	447.0927	447.0954	–	–5.882
4-methylcatechol-O-sulphate	9.7	203.0014	203.0021	123	–3.300
Pyrogallol-O-2-sulphate	3.9	204.9807	204.9814	125	–3.269
Vanillic acid-4-O-sulphate	4.2	246.9912	246.9918	167	–2.105
5-(3',4'-dihydroxyphenyl)- γ -valerolactone-4'-O-sulphate	9.5	287.0225	287.0236	207	–3.658
Dihydroxyhydrocinnamic acid-3-O-glucuronide	9.5	357.0821	357.0827	181 + 137	–1.484

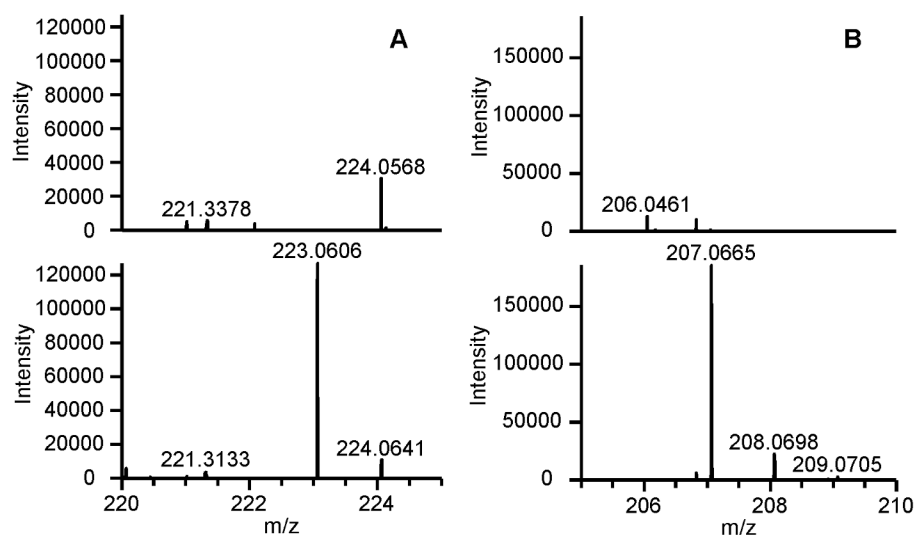


Fig. 2. Searching ions in untargeted analysis – Examples of ions not present in urine before cranberry intake (upper panels), but present in urine samples after the treatment (lower panels): ions at m/z 223.0606 and m/z 207.0665 are present only after the treatment and identified as sinapinic acid (A) and as 5-(3',4'-dihydroxyphenyl)- γ -valerolactone (B), respectively.

hydroxybenzoic acid and hydroxyhippuric acids, which presence can be related to PACs metabolism.

Phenolic acids represent the main class of identified polyphenols: protocatechuic acid, *p*-coumaric acid, gallic acid and sinapinic acid were found to be already present in the extract, but they can also derive from the metabolism of other polyphenols as mentioned above; 3,4-dihydroxyhydrocinnamic acid, dihydroxyhydrocinnamic acid-3-O-

glucuronide and 3-(4-hydroxyphenyl)-propionic acid can derive from the metabolism of PACs, chlorogenic acid or anthocyanins [17,39,46], while hydroxybenzoic acid, dihydroxybenzoic acids, hippuric acid and hydroxyhippuric acids can derive from the metabolism of all the other flavonoid components [46]. Catechol and pyrogallol derivatives, such as 4-methylcatechol-O-sulphate and pyrogallol-O-2-sulphate that we identified, can be generated from phenolic acids or anthocyanins [46].

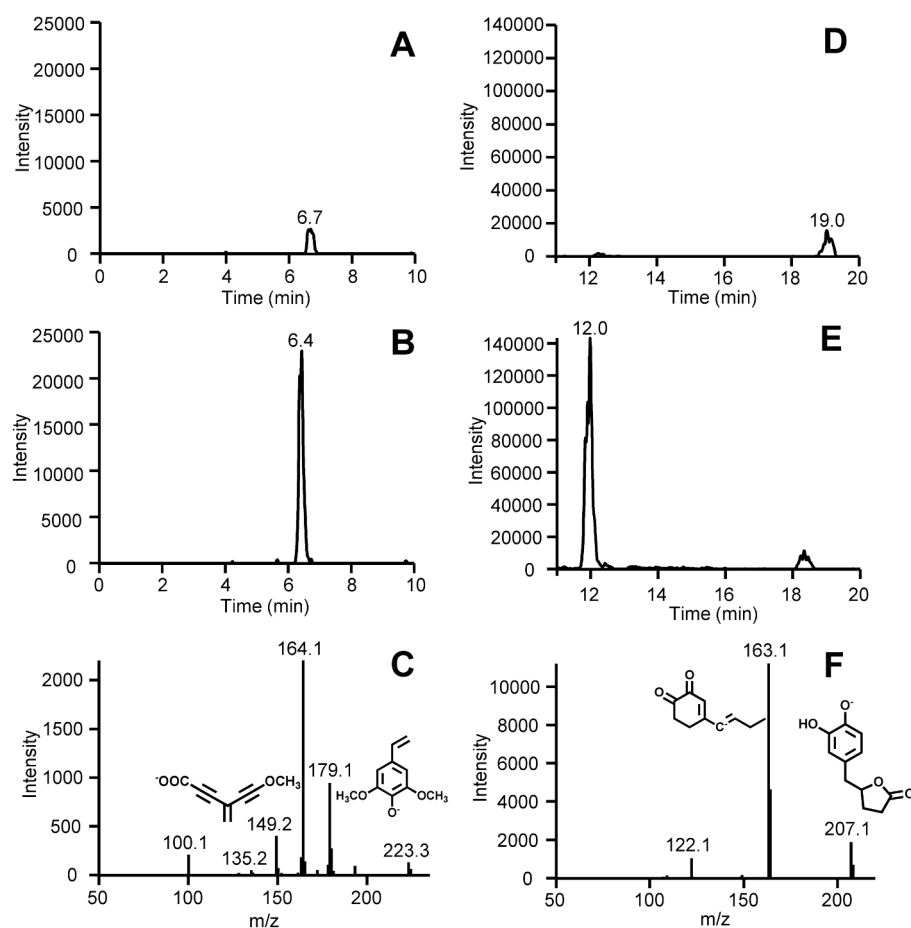


Fig. 3. Compounds identification in untargeted analysis – Single ion chromatograms relative to sinapinic acid and 5'-(3',4'-hydroxyphenyl)-γ-valerolactone (D) in urine sample before cranberry intake (panels A and D, respectively) and after 10 h and 12 h from cranberry intake (panels B and E, respectively). Peaks with the same RT of genuine standards are only detected after cranberry intake. Final confirmation of identities was achieved by tandem MS analyses. MS/MS spectra of sinapinic acid (C) and 5'-(3',4'-hydroxyphenyl)-γ-valerolactone (F) with the predicted structure for each fragment assigned by the Compound Identification tool of CFM-ID.

Table 7
Metabolites identified using the untargeted analysis. *identity confirmed by pure standard.

Metabolite putative identification	RT (min)	Calculated [M – H] [–]	Observed [M – H] [–]	MS/MS fragments	Delta ppm	Database
3,4-dihydroxyhydrocinnamic acid*	7.1	181.0499	181.0506	137 + 121	–3.811	HMDB
Quinic acid*	1.72	191.0556	191.0554	173 + 129	1.047	HMDB
2-methylhippuric acid*	11.1	192.0661	192.0670	74	–4.894	HMDB
<i>p</i> -Hydroxyhippuric acid*	3.6	194.0456	194.0450	150 + 100 + 93	3.401	HMDB
<i>m</i> -Hydroxyhippuric acid*	4.5	194.0456	194.0446	150 + 100 + 93	5.256	HMDB
<i>o</i> -Hydroxyhippuric acid (salicyluric acid)*	14.1	194.0456	194.0461	150 + 100 + 93	–2.628	HMDB
Sinapinic acid*	19.4	223.0607	223.0608	179 + 164 + 149	–0.717	HMDB
5-(3',4'-dihydroxyphenyl)-γ-valerolactone*	12	207.0657	207.0659	163 + 122 + 109	–0.821	HMDB
5-(3',4'-dihydroxyphenyl)-γ-valerolactone-3'-O-sulphate	9.5	287.0225	287.0236	207	–3.658	HMDB
5-(3',4'-dihydroxyphenyl)-γ-valerolactone-4'-O-sulphate	10.2	287.0225	287.0233	207	–2.474	HMDB
5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone-3'-O-sulphate	6.6	303.0175	303.0167	223	2.442	HMDB
4-Hydroxy-5-(dihydroxyphenyl)-valeric acid-O-sulphate	6.4	305.0331	305.0341	225	–3.213	HMDB
Dihydroxyhydrocinnamic acid-3-O-glucuronide	9.5	357.0821	357.0826	181 + 137	–1.484	HMDB
Salicyluric glucuronide	9.2	370.0774	370.0783	194 + 150	–2.513	PubChem
3-O-Methylcatechin-sulphate	12.3	383.0437	383.0442	303 + 285 + 259 + 244 + 217 + 137	–1.279	PubChem
5-(3',4'-dihydroxyphenyl)-γ-valerolactone-3'-O-glucuronide	8.1	383.0978	383.0981	207	–0.783	HMDB
5-(3',4'-dihydroxyphenyl)-γ-valerolactone-4'-O-glucuronide	9.4	383.0978	383.0982	207	–1.044	HMDB
Sinapinic glucuronide	6.8	399.0928	399.0931	223	–0.877	HMDB
5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulphoglucuronide	4.6	463.0546	463.0548	383/287/207	–0.302	[47]

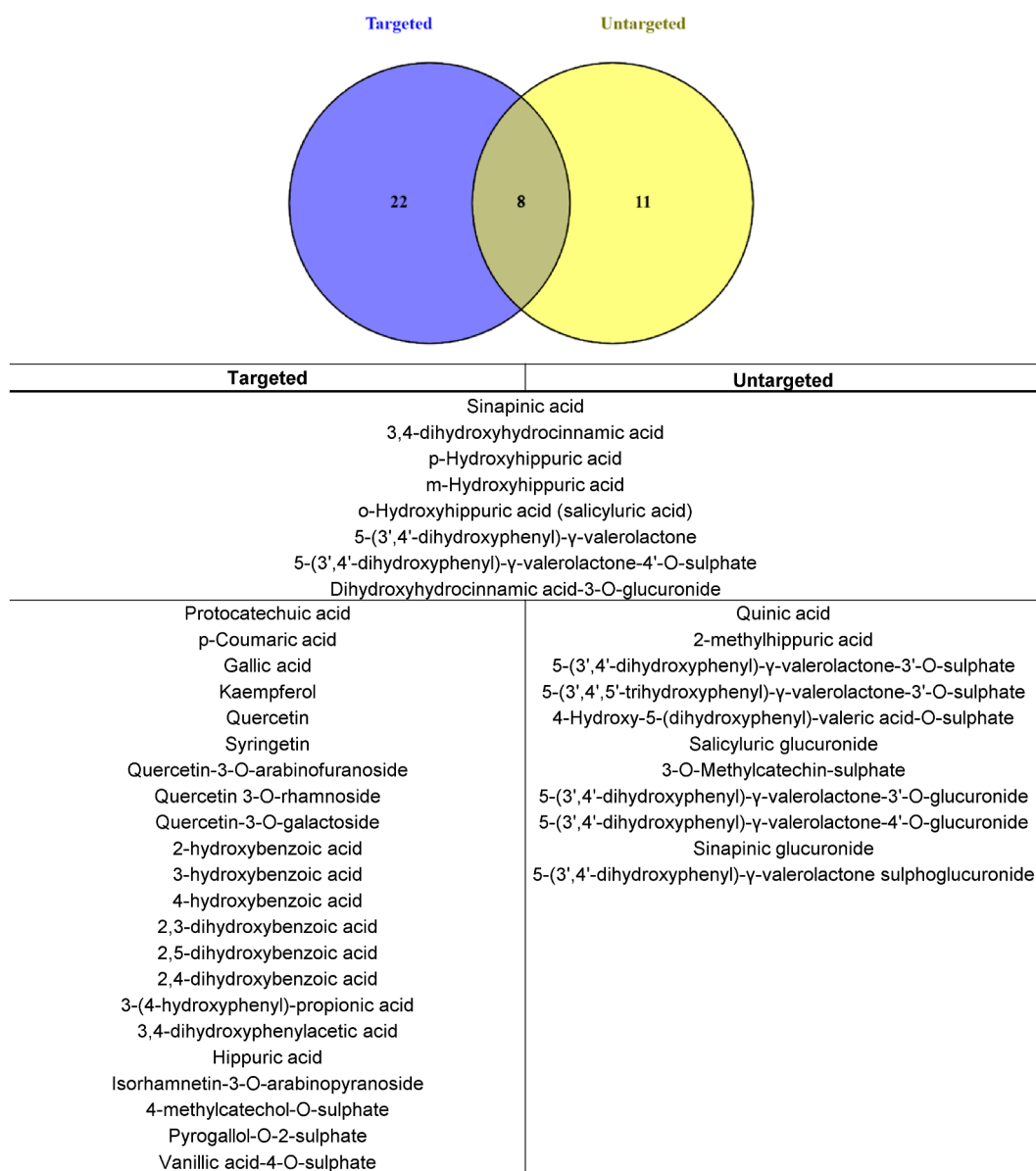


Fig. 4. Targeted and untargeted identifications – Venn diagram reporting all of the 42 compounds identified with the targeted (blue circle) and untargeted (yellow circle) analyses.

3.4. Untargeted analysis of human urine after cranberry extract intake

As described in the method session, the untargeted approach consists of searching for all the ions present in the urine samples collected after cranberry consumption that were not present or present at intensity relative to noise less than 5×10^2 counts in the pre-treatment sample. The analysis was performed using the negative ion mode because all the compounds identified using the targeted analysis were mainly detected in this polarity mode. The unidentified compounds, or those recognized as coming from the human basal metabolism (e.g. amino acids), were not included on the list. The search of ions was performed by screening the full MS spectra acquired in negative ion mode using a mass ranges of m/z 5 and with 10 min as acquisition time for each sample. As an example, Fig. 2A reports the MS spectra resulting by setting a MS range between m/z 220 and 225 and considering a time window between 0 and 10 min and relative to urine collected before (upper panel) and after 10 h (lower panel) the cranberry intake; Fig. 2B reports the MS spectra resulting by setting a MS range between m/z 205 and 210 and considering a time window between 10 and 20 min and

relative to urine collected before (upper panel) and after 12 h (lower panel) the cranberry intake. The ions at m/z 223.0606 and m/z 207.0665 are well evident only in the urine samples collected after the cranberry administration but not before. Identification of the unknown compounds was carried out by setting the precursor ion and the MS/MS fragment ions as inputs in the Compound Identification tool of CFM-ID. Fig. 3C and Fig. 3F reports the experimental MS/MS spectra used as input for the Compound Identification tool of CFM-ID which gave sinapinic acid and 5'-(3',4'-dihydroxyphenyl)-γ-valerolactone as best matched results. Final attribution was obtained by comparing RT, MS isotopic pattern and MS/MS fragmentation with those of pure standards (when commercially available). Fig. 3 shows the SIC chromatograms of sinapinic acid (Fig. 3B) and 5'-(3',4'-dihydroxyphenyl)-γ-valerolactone (Fig. 3E) in the urine fraction in which they reached their maximum concentration (10 h for sinapinic acid and 12 h for 5'-(3',4'-dihydroxyphenyl)-γ-valerolactone), while in the control sample (Figs. 3A and 3D, respectively) they were not present.

Several metabolites were identified in the untargeted analysis as reported in Table 7. Some of them had already been identified using the

Table 8
Results of known studies on metabolite characterization after cranberry products intake. The comparison regards: cranberry treatment, PACs content in the cranberry product used, the time of the treatment, the number of cranberry metabolites identified, the eventual presence of PACs and the number of known PACs metabolites including phenolic acid and valerolactone derivatives. NA: not available.

	Present study	Valentova et al. [15]	McKay et al. [20]	Walsh et al. [21]	Iswaldi et al. [16]	Feliciano et al. [17,18]	Peron et al. [19]
Cranberry Treatment	2 capsules/day standardized cranberry extract	1200 mg/day of dried cranberry juice	237 mL cranberry juice cocktail	237 mL/day cranberry juice cocktail	0.6 mL/kg of cranberry syrup	450 mL of a single-strength cranberry juice beverage	360 mg of cranberry extract
PACs content	36 mg/capsule	14.4 mg	NA	140 mg	0.71% (w/v)	710 mg	42.6% w/w of PAC-A/ 14.6% w/w of PAC-B
Treatment time	7 days	8 weeks	Single dose (24 h)	7 weeks	Single dose (24 h)	Single dose (24 h)	Single dose (24 h)
Number of metabolites	42	NA	26	19	32	67	14
PACs presence	no	no	yes	yes	no	no	no
N PACs metabolites (N valerolactones)	15 (8)	NA	8 (0)	7 (0)	3 (0)	17 (1)	12 (6)

targeted analysis, such as 3,4-dihydroxyhydrocinnamic acid, dihydroxyhydrocinnamic acid-3-O-glucuronide, sinapinic acid, the three hydroxyhippuric acid isomers, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone-4'-O-sulphate. Fig. 4 shows an overview of all the 42 compounds which have been identified in the present study with the targeted (blue circle) and untargeted (yellow circle) analyses.

Besides quinic acid, which can derive from chlorogenic acid, and 2-methylhippuric acid, a methyl derivative of hippuric acid, different valerolactones and one valeric acid derivative were identified and listed in Table 7. Among these metabolites, 5-(3'-hydroxyphenyl)- γ -valerolactone-4'-O-sulphate had been identified in previous studies [17,18]. Also 5-(3',4'-dihydroxyphenyl)- γ -valerolactone has already been reported as cranberry metabolite [19]. None of the other valerolactones here described had previously been identified in urine after cranberry intake, although they are known as PACs metabolites [39,47].

Table 8 summarizes the overall cranberry components and metabolites, including PACs and their metabolites, so far identified in human studies, in comparison with those reported in the present study. Information on the treatment (dose and PACs content of the given cranberry) are also summarized. As already discussed above, PACs were detected only in two studies [20,21] and in one of these [20] the amount of PACs in the cranberry juice was not reported. In these two studies, phenolic acids were reported as PACs metabolites and no valerolactone derivatives were detected as in the study performed by Valentova et al. [15]. Feliciano et al. [17,18] identified several PAC metabolites by using standards, and among these only one valerolactone derivative was identified, while Peron et al. [19] reported a lower amount of metabolites but a higher number of valerolactone/valeric acid derivatives, one of which was also detected in this study. Hence, the untargeted approach here reported has permitted the identification of six valerolactones/valeric acid whose presence in urine after cranberry consumption has never been described before.

3.5. Ex-vivo inhibition of *Candida albicans* biofilm-formation by urine fractions

Anthocran® (0.1 mg/mL), urine collected before administration of Anthocran®, Anthocran™ Phytosome™ or placebo and urine fractions collected after 1 h, 2 h, 4 h, 6 h, 10 h, 12 h and 24 h of each treatment were tested to investigate their potential ability to reduce *C. albicans* adhesion and biofilm formation on polystyrene 96-well plates. Anthocran® 0.1 mg/mL was able to strongly reduce the adhesion and biofilm formation ($p < 0.0001$) of the biofilm-producing strain SC5314 (data not shown). Results expressed as mean \pm SD of urine fractions are reported in Fig. 5. Urine samples before (U-Pre) each treatment were inactive, while among the seven fractions tested those collected after 12 h the Anthocran® consumption (Fig. 5A) as well as Anthocran™ Phytosome™ (Fig. 5B) were shown to significantly inhibit the adhesion compared with the control ($p < 0.05$ and $p < 0.01$, respectively for Anthocran® and Anthocran™ Phytosome™). Urine fractions after placebo intake showed no activity at all, meaning that diet and the circadian rhythm does not influence activity. It should be noted that the effect of Anthocran™ Phytosome™ at 12 hr superimposes that of Anthocran®, despite the dose of Anthocran® per capsule being 1/3 in the phytosomal preparation (12 mg PACs/capsule Anthocran™ Phytosome™ vs 36 mg PACs/capsule Anthocran®). Phytosomes are lecithin formulations demonstrated to enhance botanical ingredients oral bioavailability both at preclinical and clinical levels [48–51]. The similar ability to reduce *C. albicans* adhesion and biofilm despite the reduced cranberry extract dose can be consequently explained by considering an increased absorption of active principles allowed by the Phytosome technology. This figure can positively contribute to a more rational and convenient modulation of clinical dosage and posology.

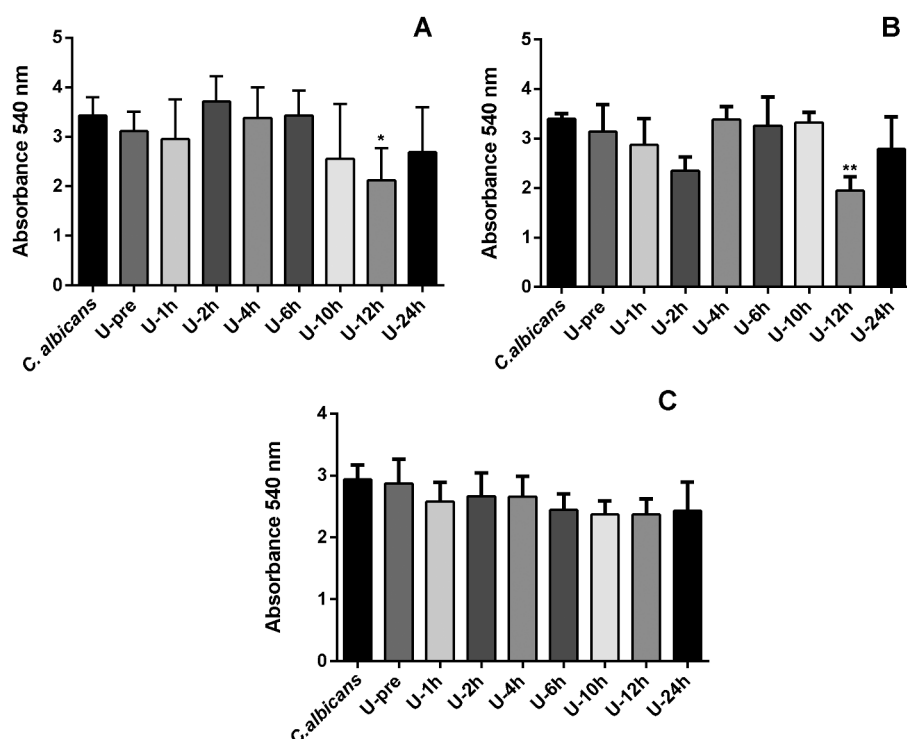


Fig. 5. Inhibition of *Candida albicans* biofilm formation – Biofilm biomass was assessed using the crystal violet assay after *C. albicans* SC5314 strain was cultured for 24 h in RPMI 1640 with/without treatments. A) Activity of urine before Anthocran® intake (U-Pre) and urinary fractions after treatment (U-1 h – U-24 h). Biomass of untreated *C. albicans* biofilm was used as control (*C. albicans*). B) Activity of urine fractions (U-1 h – U-24 h) after oral intake of Anthocran™ Phytosome™ and of urine before Anthocran™ Phytosome™ intake (U-Pre). Untreated *C. albicans* biofilm (*C. albicans*) was used as control. C) Activity of urine fractions (U-1 h – U-24 h) after oral intake of placebo (U-1 h – U-24 h). Control as described for B). Values represent the mean of three independent experiments, and of at least three sample replicates. Significant differences are indicated by *p < 0.05, **p < 0.01, ANOVA test.

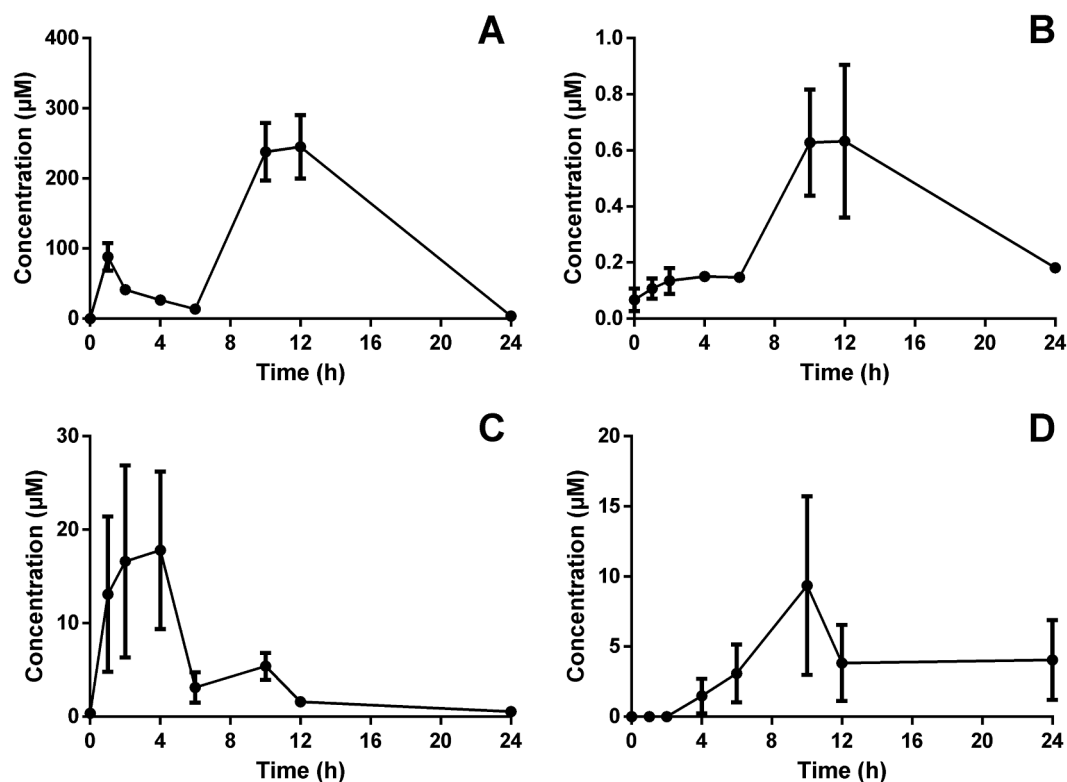


Fig. 6. Urinary profile of some metabolites – Excretion profiles of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (A), quercetin (B), p-coumaric acid (C) and 3,4-dihydroxyphenylacetic acid (D).

3.6. Metabolite concentration in urine samples

In order to identify the cranberry components/metabolites characteristic of the bioactive fractions and hence most likely responsible for the ability to reduce *C. albicans* adhesion and biofilm formation, the concentration of the available metabolites for each urinary fraction was

obtained through the quantitative analysis explained in the method section. Concentrations of the urinary components are reported as μM in order to allow the reassemble of the mixtures by using pure compounds and confirm their activities (see below). As examples, Fig. 6 shows two compounds which reached their maximum abundance in the active fraction and two compounds that reached this value in other

Table 9
Cranberry components and metabolites mean (\pm SD) concentration (μ M) in urine fractions. N.D.: not detected.

Name	Pre-treatment	1 h	2 h	4 h	6 h	10 h	12 h	24 h
Protocatechuic acid	1.20 \pm 1.00	7.24 \pm 3.31	4.97 \pm 1.52	10.81 \pm 4.45	2.12 \pm 1.33	3.78 \pm 1.47	2.03 \pm 0.82	2.51 \pm 0.94
p-Coumaric acid	0.39 \pm 0.24	13.11 \pm 8.30	16.63 \pm 10.27	17.81 \pm 8.43	3.13 \pm 1.62	5.40 \pm 1.43	1.60 \pm 0.31	0.55 \pm 0.28
Gallic acid	< LOQ	2.28 \pm 1.34	< LOQ	1.45 \pm 1.06	< LOQ	3.02 \pm 1.56	2.18 \pm 0.91	1.22 \pm 0.53
Sinapinic acid	0.29 \pm 0.19	47.02 \pm 46.73	25.36 \pm 24.52	68.25 \pm 67.50	118.75 \pm 117.77	255.90 \pm 247.79	84.29 \pm 80.26	111.53 \pm 109.32
Kaempferol	N.D.	0.083 \pm 0.033	0.04 \pm 0.03	N.D.	N.D.	0.02 \pm 0.01	0.04 \pm 0.03	N.D.
Quercetin	< LOQ	0.108 \pm 0.036	0.14 \pm 0.04	0.15 \pm 0.01	0.15 \pm 0.01	0.15 \pm 0.14	0.63 \pm 0.27	0.18 \pm 0.01
Syringetin	N.D.	0.15 \pm 0.14	0.02 \pm 0.02	N.D.	N.D.	0.15 \pm 0.14	0.03 \pm 0.02	N.D.
Quercetin-3-O-arabinofuranoside	N.D.	0.01 \pm 0.01	0.03 \pm 0.01	0.01 \pm 0.01	0.01 \pm 0.01	0.02 \pm 0.01	0.01 \pm 0.01	N.D.
Quercetin-3-O-rhamnoside	N.D.	0.04 \pm 0.02	0.03 \pm 0.01	0.02 \pm 0.01	N.D.	N.D.	N.D.	N.D.
Quercetin-3-O-galactoside	N.D.	N.D.	0.02 \pm 0.12	0.08 \pm 0.06	N.D.	N.D.	N.D.	N.D.
2-Hydroxybenzoic acid	< LOQ	0.26 \pm 0.11	< LOQ	< LOQ	< LOQ	0.85 \pm 0.44	0.01 \pm 0.01	N.D.
3-Hydroxybenzoic acid	N.D.	N.D.	N.D.	1.19 \pm 1.07	1.31 \pm 1.15	N.D.	0.36 \pm 0.15	0.38 \pm 0.18
4-Hydroxybenzoic acid	4.58 \pm 2.73	23.84 \pm 14.69	18.10 \pm 9.90	13.91 \pm 8.35	2.51 \pm 1.44	7.74 \pm 4.65	3.71 \pm 2.07	4.29 \pm 3.75
2,3-Dihydroxybenzoic acid	< LOQ	2.44 \pm 0.53	2.20 \pm 1.16	2.50 \pm 0.80	1.62 \pm 0.69	2.99 \pm 1.23	1.62 \pm 0.64	< LOQ
2,5-Dihydroxybenzoic acid	< LOQ	10.48 \pm 4.20	< LOQ	< LOQ	< LOQ	14.56 \pm 5.40	5.07 \pm 1.37	6.04 \pm 2.03
2,4-Dihydroxybenzoic acid	< LOQ	1.51 \pm 1.18	3.58 \pm 2.40	4.61 \pm 3.44	< LOQ	3.40 \pm 3.04	1.55 \pm 1.22	2.88 \pm 2.57
3-(4-Hydroxyphenyl)-propionic acid	0.58 \pm 0.55	25.79 \pm 19.77	4.22 \pm 2.85	20.23 \pm 7.92	17.74 \pm 16.76	4.10 \pm 1.91	1.18 \pm 0.64	N.D.
3,4-Dihydroxyphenylacetic acid	N.D.	N.D.	N.D.	1.49 \pm 1.24	3.10 \pm 2.06	9.36 \pm 6.36	3.85 \pm 2.70	4.06 \pm 2.85
Hippuric acid	666.15 \pm 29.07	230.12 \pm 19.82	557.72 \pm 26.45	409.82 \pm 17.03	656.46 \pm 24.06	531.84 \pm 68.23	192.91 \pm 31.13	648.52 \pm 27.88
3,4-Dihydroxyhydrocinnamic acid	0.45 \pm 0.40	4.39 \pm 4.03	2.99 \pm 2.69	3.24 \pm 2.43	2.50 \pm 2.45	7.15 \pm 4.89	4.46 \pm 2.05	5.08 \pm 2.12
p-Hydroxyhippuric acid	47.16 \pm 12.68	1.48 \pm 0.49	14.14 \pm 3.15	30.64 \pm 4.53	59.89 \pm 14.55	9.04 \pm 4.13	< LOQ	46.19 \pm 4.34
m-Hydroxyhippuric acid	24.80 \pm 13.21	21.91 \pm 3.37	37.88 \pm 12.61	25.57 \pm 8.15	43.60 \pm 10.17	32.36 \pm 3.54	29.03 \pm 13.24	24.89 \pm 3.74
o-Hydroxyhippuric acid	36.65 \pm 8.21	10.84 \pm 1.14	8.44 \pm 3.31	7.86 \pm 2.68	17.99 \pm 3.42	19.79 \pm 1.20	13.32 \pm 2.78	27.48 \pm 3.30
2-methylhippuric acid	N.D.	2.070 \pm 1.11	4.85 \pm 0.97	< LOQ	N.D.	N.D.	N.D.	N.D.
Quinic acid	5.49 \pm 3.80	12.71 \pm 8.02	6.42 \pm 4.38	10.15 \pm 8.29	9.23 \pm 2.26	17.36 \pm 9.82	14.74 \pm 7.32	8.50 \pm 4.92
5-(3',4'-dihydroxyphenyl)- γ -valerolactone	N.D.	88.34 \pm 61.63	41.51 \pm 15.10	26.40 \pm 14.03	13.83 \pm 11.89	238.22 \pm 129.46	245.24 \pm 143.59	3.83 \pm 2.08

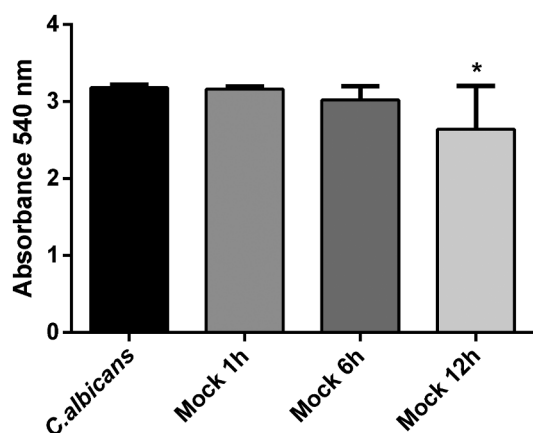


Fig. 7. Inhibition of *Candida albicans* biofilm formation by mock mixtures – The activity of the mixtures, prepared using available standards in RPMI medium, representing the inactive fractions (1 h and 6 h) and the active fraction (12 h, * $p < 0.05$) is reported.

urine fractions: Fig. 6A and B are relative to 5-(3',4'-dihydroxyphenyl)- γ -valerolactone and quercetin, respectively, whose T_{MAX} was 12 h; Fig. 6C is relative to *p*-coumaric acid, with a T_{MAX} reached after 4 h; Fig. 6D shows the excretion profile of 3,4-dihydroxyphenylacetic acid ($T_{MAX} = 10$ h). Table 9 shows the mean cranberry metabolite concentrations (μ M) in the control and in the urine fractions after cranberry intake.

The mixture of the active urine fractions (12 h) and of two inactive fractions (1 h and 6 h) were then reassembled by using available standards dissolved in RPMI and tested on *C. albicans*. The 12 h mixture was found to significantly reduce adhesion while the other two mixtures were inactive, thus confirming the results of the ex-vivo urine samples (Fig. 7). It is important to underline that the 12 h reconstituted mixture only contained one of the 8 valerolactone derivatives identified (namely, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone) due to the lack of commercial availability of valerolactone standards (their synthesis is on-going in our laboratories). Hence if the bioactivity is related to this class of compounds, as expected, a greater activity could be obtained by integrating the reconstituted mixture with the other seven valerolactones.

The antiadhesive properties of cranberry, both *in vitro* and *in vivo*, have long been reported [52–55] mainly with regard to *E. coli*, which is the principal uropathogen that causes UTIs. An *in vitro* study showed that cranberry PACs prevent *C. albicans* biofilm formation in artificial urine [56]. In the present work, we demonstrate for the first time that cranberry extract as well as some urine fractions, collected after one week of cranberry administration, reduce both *C. albicans* adhesion and biofilm biomass. Since 12 h urine fractions were the most active, we focused our attention on the components that reached their highest concentration at this time point. The components found to have the highest concentration in this urine fraction are quercetin and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone. A recent *in vitro* study [57] demonstrates that valerolactone derivatives display anti-adhesive activity against *E. coli*, confirming that the *in vivo* activity is due to PACs metabolites rather than intact PACs. Moreover, quercetin was reported to have *in vitro* anti-adhesive properties on *E. coli* [58]. However, the activity could derive not only by a single component but from a synergy of all the metabolites present in that mixture, thus explaining the possible activity of cranberry as a phytocomplex. In fact, many components present in the mixture showed an activity against biofilm formation or anti-adhesive properties against *E. coli*, like protocatechuic acid, 3,4-dihydroxyphenylacetic acid and 2-hydroxybenzoic acid [59–61].

In conclusion, the HR-MS method developed allowed the identification of several cranberry components and metabolites in human urine after a highly standardized cranberry extract consumption which has

been found to be effective in human studies. PACs were not detected as reported by previous studies, but several metabolites deriving from their catabolism presumably operated by the gut microflora and hereto not found *in vivo* were identified. The crude extract and the urine fraction collected at 12 h after cranberry intake were found to be active against *C. albicans* adhesion *ex-vivo*. The known metabolite of PACs, 5-(3',4'-dihydroxyphenyl)- γ -valerolactone was identified as the most abundant metabolite (245 μ M) in the bioactive urine fraction. To our knowledge, this is the first work which demonstrates an *ex-vivo* inhibition of *C. albicans* adhesion by human urine after cranberry intake. As a future perspective, as soon as all the identified compounds peaking at 12 h will be available (the synthesis of valerolactones derivatives is on-going) their activity against *C. albicans* will be evaluated, with a particular interest focused on valerolactone derivatives which represent the most abundant metabolites in urine after cranberry intake. Furthermore, in order to understand whether a synergistic action is involved, compounds will be tested in pure form or in mixture.

A further interesting result of our study derives from the use of the lecithin formulation of the cranberry extract which has shown to markedly increase oral bioavailability and organ target accessibility of cranberry active principles. A clinical study in catheterized subjects is on-going to confirm the effectiveness of Anthocran™ Phytosome™.

Author contributions

GB and GA were the principal responsible for the experimental part and for writing the manuscript. PA, GP, PM AR, MC, GB and GA conceived and designed the study. LF and A. Artasensi contributed to the synthesis of valerolactone derivative. EB and EO contributed to the microbiological studies. CDB and PR contributed to the design of the human volunteers study. GB, AA, LC, LA performed the analyses. All the authors assisted with the manuscript preparation.

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Conflict of interest and sponsorship

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